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A novel enzymatic pathway leading to 1-methylinosine modification in *Haloferax volcanii* tRNA

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ABSTRACT

Transfer RNAs of the extreme halophile *Haloferax volcanii* contain several modified nucleosides, among them 1-methylpseudouridine ($m^1\psi$), pseudouridine (ψ), 2′-0-methylcytosine (Cm) and 1-methylinosine (m^1I), present in positions 54, 55, 56 and 57 of the ψ-loop, respectively. At the same positions in tRNAs from eubacteria and eukaryotes, ribothymidine (T-54), pseudouridine (ψ-55), non-modified cytosine (C-56) and non-modified adenosine or guanosine (A-57 or G-57) are found in the so-called Tψ-loop. Using as substrate a T_7 transcript of *Haloferax volcanii* $tRNA^{Ile}$ devoid of modified nucleosides, the enzymatic activities of several tRNA modification enzymes, including those for $m^1\psi$-54, ψ-55, Cm-56 and m^1I-57, were detected in cell extracts of *H.volcanii*. Here, we demonstrate that modification of A-57 into m^1I-57 in *H.volcanii* $tRNA^{Ile}$ occurs via a two-step enzymatic process. The first step corresponds to the formation of m^1A-57 catalyzed by a S-adenosylmethionine-dependent tRNA methyltransferase, followed by the deamination of the 6-amino group of the adenine moiety by a 1-methyladenosine-57 deaminase. This enzymatic pathway differs from that leading to the formation of m^1I-37 in the anticodon loop of eukaryotic $tRNA^{Ala}$. In the latter case, inosine-37 formation preceeds the S-adenosylmethionine-dependent methylation of I-37 into m^1I-37. Thus, enzymatic strategies for catalyzing the formation of 1-methylinosine in tRNAs differ in organisms from distinct evolutionary kingdoms.

INTRODUCTION

Post-transcriptional maturation of tRNAs is a complex enzymatic process leading to restructuration of the primary pre-tRNA transcript, including numerous base modifications and occasional base conversions (tRNA editing). The role of the modified nucleosides is the subject of intensive investigations. Some of them appear to be related to specific biological functions such as protection against nucleases, translation of the genetic code, specific recognition of tRNA by aminoacyl-tRNA synthetases and/or elongation factors or stabilization of the tRNA architecture (for recent reviews see refs 1,2).

Studies on archaeal tRNA from halophiles and thermophiles have revealed several modified nucleosides that are unique to archaea (3–13, compiled in 14). A few of these, like 1-methylpseudouridine ($m^1\psi$)(7), 3-(β-D-ribofuranosyl)-4,9-dihydro-4,6,7 trimethyl-9-oximidazol (1,2-α) purine (mimG)(9), archaeosine (2-amino-4,7-dihydro-4-oxo-7-β-D-ribofuranosyl-1*H*-pyrrolo[2,3-*d*] pyrimidine-5-carboximidamide) (12), 3-hydroxy-*N*-{[(9-β-D-ribofuranosyl-9*H*-purin-6-yl) amino] carbonyl} norvaline (hn^6A) and its 2-methylthio derivative ms^2hn^6A (13) have no equivalent in eukaryotic or eubacterial tRNAs. Other characteristic nucleosides in tRNAs from archaea are modified both in the base and by methylation of the 2′ hydroxyl group in ribose, like m^5Cm, m^1Im, ac^4Cm, s^2Um, m^2Gm and $m^2{}_2Gm$ (6,10) whereas only the base is modified in corresponding nucleosides derived from eukaryotic and eubacterial tRNAs (4,5). These extra methylations may be important as stabilizing factors of the tridimensional structure of tRNAs (15,16) in those microorganisms which live at temperatures as high as 110°C or in very high salt media. While these modified nucleosides were identified in tRNA hydrolyzates, their locations in archaeal tRNA sequences are not known.

Among archaea, tRNAs from *Haloferax volcanii* have been the most extensively studied (17,18, reviewed in 19) and account for 41 of the 59 archaeal tRNA sequences reported so far (20). The location and frequency of occurrence of the 14 different kinds of modified nucleosides identified in *H.volcanii* tRNAs are shown in Figure 1a. Only those indicated by black circles have no equivalent in terms of their chemical structure and/or their location in any of the tRNAs sequenced so far from eubacteria or eukaryotic cells (21). Among those nucleoside modifications unique to archaea, 1-methylpseudouridine-54 ($m^1\psi$–54) and 2′-O-methylcytosine-56 (Cm-56) in the ψ-loop are present in all the 41 *H.volcanii* tRNAs, whereas adenosine-57 is always modified to 1-methylinosine-57 (m^1I-57), except in the case of $tRNA^{His}$ (17). All the other *H.volcanii* tRNAs present a guanosine-57 instead of A-57 or m^1I-57 (17,18). The three modified nucleosides $m^1\psi$-54, Cm-56 and m^1I-57 are also present in tRNAs from other halophilic archaea (*Halobacterium cutirubrum* and *Halococcus morrhuae*) (22,23), while Cm-56 and m^1I-57 were also found in tRNA sequences from thermophiles and methanogens (*Sulfolobus acidocaldarius, Thermoplasma acidophilum* and *Methanobacterium thermoautotrophicum*) (6,24,25).

At present, nothing is known about the biosynthesis of the modified nucleosides in archaebacterial tRNAs. Only one report

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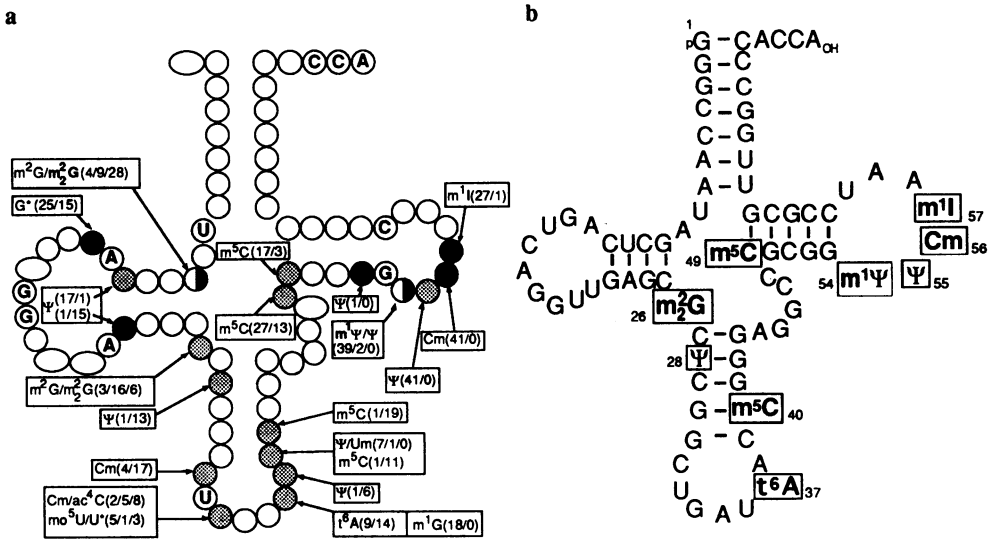

Figure 1. Occurrence of modified nucleosides in tRNAs from *H.volcanii*. (**a**) Composite secondary structure of 41 tRNAs from *H.volcanii* (as cited in ref. 20). Positions of the modified nucleosides are indicated by arrows in the cloverleaf structure, the conventional abbreviations (symbols as in ref. 14) are used, except for G*(archaeosine) and U* (unknown modified uridine). The numbers in brackets next to each symbol indicate the frequency of the modified nucleoside versus the number of the unmodified one (for example, 4-fold m^2G, 9-fold m^2_2G and 28-fold unmodified G in position 10; or 27-fold m^1I and one unmodified A in position 57) (see also ref. 21). Black circles correspond to positions in tRNA where the indicated modified nucleoside is characteristic of archaea. Half black circles correspond to positions where only one of the two indicated modified nucleosides is characteristic of archaea (m^2_2G at position 10 and $m^1\psi$ at position 54). Grey circles correspond to positions where the indicated modified nucleosides are also present in one or several tRNAs from eukaryotes and/or eubacteria. Open circles correspond to positions where no modified nucleoside was found in any of the tRNAs from *H.volcanii* sequenced so far. (**b**) Secondary structure of the $tRNA^{Ile}$ of *H.volcanii*. Numbers outside the boxes correspond to conventional numbering or positions as in ref. 20. Each of the nine modified nucleosides present in this tRNA are boxed. Only $m^1\psi$-54, Cm-56 and m^1I-57 correspond to modified nucleosides that are unique to date to archaea.

(26) describes the methylation of total tRNAs by *Methanococcus vannielii* extracts, using S-adenosylmethionine as the methyl donor and undermethylated *E.coli* tRNAs as the substrate. In the present study we have developed an *in vitro* system that allowed us to measure the activities of the enzymes catalyzing the formation of several modified nucleosides of an halophilic tRNA. In particular, we show that the enzymatic formation of 1-methylinosine found at position 57 of several *H.volcanii* tRNAs (27) differs from that previously demonstrated for 1-methylinosine located at position 37 in the anticodon loop of eukaryotic $tRNA^{Ala}$.

MATERIALS AND METHODS

Strains and DNA plasmids

The *H.volcanii* strain DS2 (NCMB 2012; ATCC 29605) was kindly provided by Prof. P. Forterre (University Paris XI, Orsay, France). The plasmid pUC19 containing the synthetic $tRNA^{Ile}$ gene (with G-57) of *H.volcanii* was a gift from Dr C.J. Daniels (Ohio State University, Colombus, OH). Procedures identical to the ones described earlier for the construction of the synthetic *H.volcanii* $tRNA^{Trp}$ gene were used for construction of the $tRNA^{Ile}$ gene (29). It contains a T_7 promoter upstream of the tRNA sequence and a linearizing *Mva*I nuclease restriction site downstream of the gene. The $tRNA^{Ile}$ gene, together with the T_7 promoter and the *Mva*I nuclease restriction site, was subcloned in the pUC118 plasmid. This allowed mutagenesis of G-57 to A-57 from the single-stranded DNA using the oligonucleotide-directed mutagenesis system (Amersham) and *E.coli* strain TG1 *sup* E, *hsd*Δ5, *thi*, Δ(*lac-pro*AB), F′(*tra*D36, *pro*AB+, *laq*I^q, *lac*ZΔM15) as recepient for plasmid transformations.

Preparation of *H.volcanii* radiolabelled $tRNA^{Ile}$ substrate

The *H.volcanii* $tRNA^{Ile}$, lacking all the nucleoside modifications, was prepared by *in vitro* transcription using T_7 RNA polymerase, following the procedure described in ref. 30. Prior to transcription, the plasmid (10 μg) containing the $tRNA^{Ile}$ gene was digested with 30 U *Mva*I nuclease (Boehringer) in 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 100 mM NaCl and 1 mM DTE (buffer H, provided by the manufacturer). The completeness of digestion was tested by electrophoresis on 1% agarose gel. After phenol-chloroform extraction and ethanol precipitation, 1 μg of the digested plasmid was lyophilized with 50 μCi of one of the four [α-^{32}P] nucleoside triphosphates obtained from Amersham (sp. act. 400 Ci/mmol). The lyophilized product was resuspended in 10 μl of reaction mixture containing 40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT (Sigma), 2 mM spermidine (Sigma), 1 mM of each NTP except for the labelled one (100 μM final concentration), 10 U RNasin (Promega), 8 U T_7 RNA polymerase (Promega). The mixture was incubated for 2 h at 37°C. The reaction was stopped with 0.1 vol EDTA 0.5 M and 10 μl of the mixture: 8 M urea, 30% sucrose, 0.1% bromophenol blue, 0.5% xylene cyanol. The radiolabelled sample was loaded on a 6% denaturing polyacrylamide-urea gel and subjected to electrophoresis for 1 h at 500 V. After autoradiography, the radiolabelled transcript (~50–150 pmol, having incorporated ~5–15 μCi of the radiolabelled nucleotide) was recovered from the gel by overnight elution with a salt

solution containing 0.5 M ammonium acetate buffer at pH 5.0, 10 mM magnesium acetate, 0.1 mM EDTA, 0.1% (w/v) SDS. The eluted RNA was ethanol-precipitated at –20°C, washed twice with cold ethanol and redissolved in water.

Preparation of *H.volcanii* S100 extract

H.volcanii cells were grown aerobically at 37°C from a preculture with gentle agitation in a classical halophilic medium (500 ml) as described in ref. 31. After 48 h, pink-red cells were harvested by centrifugation at 5000 *g* for 15 min, washed once with a cold solution containing 3.6 M NaCl, 150 mM $MgSO_4$, 1 mM $MnCl_2$, 50 mM Tris-HCl pH 7.5, 3.4 mM $CaCl_2$ and resuspended at 0°C in 5-fold their weight of 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 2% glycerol, 2.2 M KCl, 2 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM benzamidine, 10 mg/l leupeptin, 10 mg/l antipaïne, 10 mg/l chymostatine, 10 mg/l pepstatine A (all antiprotease compounds were from Sigma). Cells (usually 4 g) were disrupted at 4°C by sonication in a Braun 12TL sonicator, 4 × 30 s. Cell debris and the macromolecular components (mainly ribosomes) were removed by ultracentrifugation at 100 000 *g* for 1 h at 4°C in a Beckman TL-100 ultracentrifuge. The S100 clear supernatant was concentrated to 2 or 3 ml at ~25 or 30 mg/ml by centrifugation at 3000 *g* in an Amicon centriprep (cut off 10 kDa). The resulting concentrated solution was diluted with 1 ml of 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 2% glycerol, 2.2 M KCl, 2 mM DTT and concentrated again with a centriprep as above. After repeating this step 2-fold more, the concentrated S100 extract was stored at 4°C until use. No significant loss of enzyme activity was observed over a period of 1 month. Its protein content was determined according to Bradford (32), using the dye reagent of Bio-Rad Laboratories.

Further purification of the *H.volcanii* S100 extract

The above S100 extract (0.5 ml, ~15 mg proteins) was dialyzed overnight against buffer 1 containing 2.2 M $(NH_4)_2SO_4$, 50 mM Tris-HCl (pH 7), 5 mM β-mercaptoethanol. The clear dialyzate was applied to a 5 ml column of Sepharose 4B (Pharmacia), preequilibrated with buffer 1 at room temperature according to Mevarech *et al.* (33). The column was washed with 50 ml of buffer 1 and the proteins that flowed through the column were pooled (Fraction A). The adsorbed proteins (Fraction B) were then eluted from the column with buffer 2 containing 0.5 M $(NH_4)_2SO_4$, 50 mM Tris-HCl (pH 7), 5 mM β-mercaptoethanol. Fractions A and B were extensively dialyzed against the same buffer containing 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 2% glycerol, 2.2 M KCl, 2 mM DTT. Each dialyzate was concentrated to ~0.5 ml with Amicon centripreps (cut off 10 kDa) and the protein content was determined as above.

Enzyme assay

The standard assay (total vol 50 µl, including the enzyme) contained 100 mM Tris-HCl (pH 8), 10 mM $MgSO_4$, 0.1 mM EDTA, 100 mM ammonium acetate, 2 mM DTT, 75 µM S-adenosylmethionine (SAM, obtained from Boehringer), 2.2 M KCl, 13 U RNasin (Promega) and ~5×10^4 c.p.m. $tRNA^{Ile}$ (~40 fmol tRNA). A concentration of 2.2 M KCl in the reaction medium, the same as for disrupting the *H.volcanii* cells, was arbitrarily used. This may not be the optimal salt concentration for each of the different enzymes tested. The reaction was started by the addition of S100 extract (final protein concentration in the assay: 15 or 20 mg/ml) or of fraction A (~0.8 mg protein/ml) or fraction B (~8 mg protein/ml). After various times of incubation at 37°C, the proteins were extracted with phenol-chloroform and the tRNA precipitated twice with ethanol in the presence of 0.3 M sodium acetate. Radiolabelled tRNA was dissolved either in 50 mM ammonium acetate pH 5.3 and hydrolyzed into nucleoside 5′-monophosphates with 2 µg of nuclease P_1 (from Boehringer) or in 50 mM ammonium acetate pH 4.6 and hydrolyzed into nucleoside 3′-monophosphates with 0.1 U of nuclease T_2 (from Sigma), in a total reaction vol of 10 µl.

Identification and quantitation of the enzyme products

Identification of modified nucleotides was performed by two-dimensional thin layer chromatography (TLC) on 10 × 10 cm CEL 400 M cellulose plates (Schleicher and Schuell). The TLC plates were developed in solvent systems composed of isobutyric acid :25% NH_4OH:H_2O (66:1:33 by vol) in the first dimension and in the second dimension either 0.1 M sodium phosphate pH 6.8: ammonium sulfate: *n*-propanol (100:60:2, v:w:v) for system I or isopropanol:37% hydrochloric acid:H_2O (68:17.6:14.4 by vol) for system II. After autoradiographies, the identification of radiolabelled spots was performed by comparison with reference maps (17,34). Evaluation of the mol of modified residues/mol of tRNA was done by counting the radioactivity of each spot, normalized by the number of A, C, G and/or U residues in the whole $tRNA^{Ile}$ molecule (for details, see ref. 35).

RESULTS

Analysis of the modified nucleosides in tRNA

Figure 1b shows the nucleotide sequence of *H.volcanii* $tRNA^{Ile}$ (anticodon GAU). It contains nine modified nucleosides of which only pseudouridine (ψ) in position 28 and 5-methylcytosine (m^5C) in position 40 are unique to this particular $tRNA^{Ile}$. The seven other positions contain modified nucleosides ($m^2$2G-26, t^6A-37, m^5C-49, m^1ψ-54, ψ-55, Cm-56 and m^1I-57) that are also found in several other tRNA species of *H.volcanii* (compare Fig. 1a and b). Among these, only m^1ψ-54, Cm-56 and m^1I-57 in the ψ-loop of *H.volcanii* $tRNA^{Ile}$ are characteristic of archaeal tRNAs (20,21).

To test the activities of the enzymes that catalyze the formation of these modified nucleosides *in vitro*, a T_7-transcript of a synthetic tRNA gene having the nucleotide sequence of *H.volcanii* $tRNA^{Ile}$ was used as substrate. Detection of each individual modified nucleoside formed in $tRNA^{Ile}$ was facilitated by using T_7-transcripts separately radiolabelled with each of the three canonical [α-^{32}P] nucleoside triphosphates (ATP, CTP or UTP). After incubation with the enzyme extract, each of the differently labelled tRNAs was phenol-ethanol-extracted, then hydrolyzed either with nuclease P_1 or with RNase T_2. Nuclease P_1 generates 5′-nucleoside monophosphates, of which only the nucleotide used for the labelling of the tRNA transcript (or its modified counterpart) contains [^{32}P]-phosphate, while RNase T_2 generates 3′-nucleoside monophosphates of which only the nucleotide 5′ adjacent to the one used for the labelling (or its modified counterpart) contains [^{32}P]-phosphate. Both types of hydrolyzates were analyzed by two-dimensional chromatography on thin layer cellulose plates (2D-TLC), using solvent systems I and/or II, as described in Materials and Methods. In this way, not only each type of modified nucleoside in $tRNA^{Ile}$ can be identified but also its 3′-adjacent

neighbouring nucleoside. Because m^5C-40 and m^5C-49 both have a 3′-adjacent guanosine residue in *H.volcanii* $tRNA^{Ile}$, the position from which m^5C predominantly originates cannot be evaluated by just testing the nucleotide composition of the whole $tRNA^{Ile}$ molecule. A similar situation prevails for ψ-28 and ψ-55, which both have a 3′-adjacent cytosine residue (Fig. 1b). One way to overcome this problem would be to digest first the $tRNA^{Ile}$ with RNase T_1, separate the resulting oligonucleotide fragments by gel electrophoresis or column chromatography and analyze their nucleotide composition as described above. Indeed, m^5C-40, m^5C-49, ψ-28 and ψ-55 are present in RNaseT_1-fragments of different sizes (7, 4, 6 and 10 nucleotides in length, respectively). In the present work, we have analyzed the nucleoside composition only of the whole $tRNA^{Ile}$, not of T1-oligonucleotide fragments.

Table 1 shows the results and Figure 2 (a–d) illustrates some of the 2D-TLC obtained. In these experiments the T_7 $tRNA^{Ile}$ transcript was incubated for 6 h at 37°C with a S100 extract of *H.volcanii* in 2.2 M KCl and in the presence of SAM as donor of methyl groups. Clearly, the different types of modified nucleoside monophosphates normally present in the halophilic archaebacterium *H.volcanii* $tRNA^{Ile}$ as shown in Figure 1b, are present in the tRNA hydrolyzates, with the exception of N^6-threonylcarbamoyladenosine monophosphate (t^6A). This may be because the necessary cofactor(s) for the formation of this hypermodified nucleoside, probably an activated form of carbamoylthreonine, was missing in our incubation mixture. On the other hand a pseudouridine (ψ) 5′-adjacent to an uridine (Fig. 2b) and 1-methyladenosine (Fig. 2a), was also found (see explanations below). The yields of modified nucleosides formed, as expressed in mol/mol of tRNA (Table 1), are between 0.2 and 0.8. These yields could probably be improved by optimizing experimental conditions, in particular the concentration and type of salts in the reaction mixture.

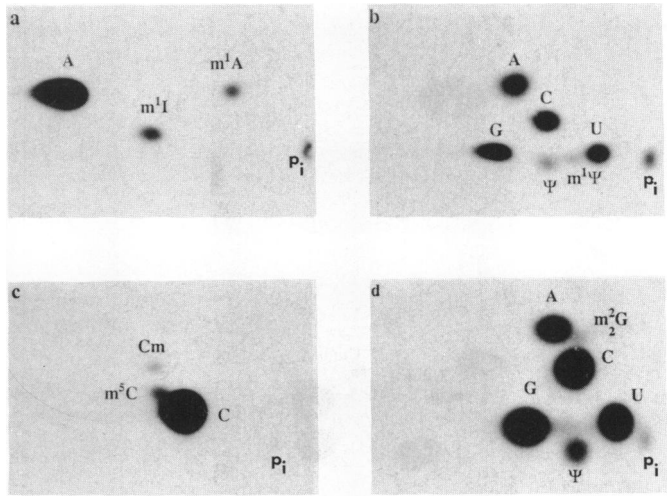

Figure 2. Modified nucleosides formed in $tRNA^{Ile}$ transcript incubated for 6 h at 37°C with *H.volcanii* S100 extract. SAM and 2.2 M KCl were present in the incubation mixture. Presence of modified nucleosides in tRNA was analyzed as described in Materials and Methods. In the figure are few selected autoradiograms of thin layer plates after chromatography in two dimensions. The experimental strategy for detecting the modified nucleotides is as indicated in Table 1 and as described in Materials and Methods. In (**a**) labelling was with [α-^{32}P]-ATP, hydrolysis with nuclease P_1 and use of chromatographic system I; in (**b**) labelling was with [α-^{32}P]-UTP, hydrolysis with RNase T_2 and chromatography in system II; in (**c**) labelling was with [α-^{32}P]-CTP, hydrolysis with nuclease P1 and chromatography in system I; in (**d**) labelling was with [α-^{32}P]-CTP, hydrolysis with RNase T_2 and chromatography in system II. Pi, inorganic phosphate. Symbols for modified nucleotides are as in ref. 14.

Origin of ψ, 5′-adjacent to an U

The only position in $tRNA^{Ile}$ where such a pseudouridine could occur is at position 54, where the expected $m^1\psi$ was present (compare Fig. 1a and 1b). This ψ therefore corresponds to the intermediate product of a stepwise order of modification of U-54 into $m^1\psi$-54. The methyl group in $m^1\psi$ occupies the normal position of the N-glycosidic bound in the uridine, obviously uracil has to rotate to form pseudouridine, prior to methylation at N-1 (7).

Table 1. Modified nucleosides detected on a synthetic $tRNA^{Ile}$ substrate with *H.volcanii* crude extract as the source of enzymes

Modified nucleoside	Position in $tRNA^{Ile}$	Mol/mol $tRNA^{Ile}$	Nuclease used for hydrolysis	Radiolabelled nucleotide	TLC system
$m^2{}_2G$	26	0.2	RNase T_2	[α^{32}P]-CTP	II
ψ	28, 55	0.7	RNase T_2	[α^{32}P]-CTP	II
ψ	28, 54, 55	1.4	nuclease P1	[α^{32}P]-UTP	II
t^6A	37	0.0	nuclease P_1	[α^{32}P]-ATP	I
m^5C	40, 49	0.8	nuclease P_1	[α^{32}P]-CTP	I
$m^1\psi$	54	0.1	RNase T2	[α^{32}P]-UTP	II
ψ	54	0.5	RNase T_2	[α^{32}P]-UTP	II
Cm	56	0.3	nuclease P_1	[α^{32}P]-CTP	I
m^1I	57	0.4	nuclease P_1	[α^{32}P]-ATP	I
m^1A	57	0.3	nuclease P_1	[α^{32}P]-ATP	I

Incubation was for 6 h in the conditions described in Materials and Methods. Strategy for evaluation of the modified nucleosides in mol/mol of tRNA were as in the text and Materials and Methods. These values are are given with an approximation of ± 0.1. Thin layer chromatographic systems I or II are explained in Materials and Methods. Symbols for modified nucleosides are as in ref. 14.

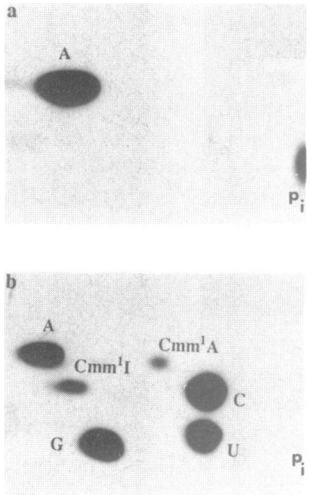

Figure 3. Autoradiography of chromatograms of $tRNA^{Ile}$ hydrolyzates. Experimental conditions are the same as in Fig. 2, except that in (**a**) a synthetic [α-^{32}P]-ATP-labelled $tRNA^{Ile}$, mutagenized in position A-57 to G-57, was used; in (**b**) the wild type $tRNA^{Ile}$ (having A-57) was used and digested with RNase. For both chromatograms, solvent system I was used. Cmm^1I and Cmm^1A correspond, respectively, to dinucleoside diphosphates between 2′-O-methylcytosine (Cm) and 1-methylinosine (m^1I) or 1-methyladenosine (m^1A).

Origin of m^1A

The unexpected modified nucleotide found on TLC was 1-methyladenosine monophosphate (m^1A in Fig. 2a, and Table 1). From inspection of the tRNA sequences available from archaeal tRNAs, this nucleoside naturally occurs in position 58 (20,21) but has never been found in any of the 41 tRNAs of *H.volcanii* sequenced so far (17,18). We suspected therefore that this nucleoside may arise as an intermediate in the formation of 1-methylinosine at position 57. In order to investigate this point, the synthetic $tRNA^{Ile}$ gene was mutagenized *in vitro* to obtain G-57 instead of A-57. About half of the tRNAs in *H.volcanii* contains such a G-57 in a fairly conserved ψ-loop sequence (17,18). Incubation of the ^{32}P-labelled T_7-transcript of this mutant $tRNA^{Ile}$ gene with *H.volcanii* S100 extract, gave no m^1IMP nor m^1AMP on the TLC plates after hydrolysis with nuclease P_1 (Fig. 3a). On the other hand, when the wild type [^{32}P]-ATP-labelled $tRNA^{Ile}$ (with A-57) was hydrolyzed with RNase T_2, two characteristic radioactive spots appeared on the TLC, one corresponding to the dinucleoside diphosphate with Cm and m^1A and the other to the dinucleoside diphosphate with Cm and m^1I (Fig. 3b). The only way to explain this result is that m^1A is indeed formed at the same position in $tRNA^{Ile}$ as m^1I-57, that is 5′ adjacent to Cm-56, the only Cm found in naturally occurring *H.volcanii* $tRNA^{Ile}$.

m^1A is an intermediate in m^1I-57 formation

It is important to note that no trace of inosine in tRNA was ever detected in our assays with *H.volcanii* extracts, even when the $tRNA^{Ile}$ was incubated in the absence of SAM. This was an unexpected observation, since inosine was clearly shown to be the obligate intermediate in the formation of m^1I-37 from A-37 in the anticodon loop of a T_7-$tRNA^{Ala}$ transcript when incubated with yeast or rat liver S100 extracts (28). One key experiment supporting this conclusion was that when the latter assays were performed in the absence of SAM, the characteristic inosine-37 was formed. Moreover, this I-37 could be subsequently transformed quantitatively into m^1I-37 upon addition of SAM to the reaction medium. In no case was the formation of m^1A-37 observed in such an eukaryotic tRNA-modification system (28, C. Simon, unpublished results). The only m^1A that could be formed in eukaryotic tRNAs is located either at position 14 or at position 58 (compiled in refs 20,21).

Figure 4a shows the time course of m^1A and m^1I formation in the T_7-$tRNA^{Ile}$ transcript when SAM was added in the reaction mixture prior to the addition of *H.volcanii* S100 extract. Figure 4b shows the same experiment but with the SAM added after 2 h incubation with the S100 extract. Clearly, both m^1A and m^1I always appear simultaneously in $tRNA^{Ile}$. Notice that the sum of the two modified nucleosides never exceeds 1 mol/mol tRNA, which is consistent with the fact that the two modifications concern the same position 57 in $tRNA^{Ile}$. From these experiments, we concluded that: (i) m^1A-57 is an obligatory intermediate of the reaction leading to m^1I-57; (ii) contrary to m^1A-37 in eukaryotic $tRNA^{Ala}$ (28), enzymatic formation of inosine-57 from adenosine in *H.volcanii* $tRNA^{Ile}$ (probably a deaminase) is a SAM-dependent process.

Two separable enzymes are required to catalyze m^1I-57 formation

Most halophilic proteins are known to adsorb on Sepharose 4B in the presence of high salt such as 2.2 M ammonium sulfate. The adsorbed proteins can be desorbed by decreasing the salt in the elution buffer (32). Using this procedure, we have attempted to partially purify the enzymatic activity(ies) responsible of m^1I-57 formation. With this aim, the S100 extract from a fresh culture of *H.volcanii* was applied to a Sepharose 4B column. A first protein fraction (fraction A) that does not adsorb on the chromatographic support, was eluted with a 2.2 M ammonium sulfate buffer. A second protein fraction (fraction B) was obtained by step elution of the column with ammonium sulfate at a concentration of 0.5 M (results not shown).

Using [^{32}P]-ATP labelled $tRNA^{Ile}$ as substrate, the presence of enzymatic activities for m^1I and/or m^1A modifications was tested in fractions A and B, singly or in combination. No enzymatic activity was detected with fraction A (Fig. 5a), whereas significant m^1A modification and only trace amounts of m^1I (not easily seen on the TLC autoradiogram) were obtained with fraction B (Fig. 5b). Interestingly, the mixture of fractions A and B catalyzed the formation of m^1I as well as m^1A (Fig. 5c). These results clearly demonstrate that a SAM-dependent tRNA(A-57) methyltransferase was present in fraction B. Concerning the enzyme catalyzing the formation of m^1I-57 from m^1A-57, it may be present either in fraction A or in fraction B. The latter possibility would be accounted for by assuming that a macromolecular component from fraction A is required to activate the enzyme present in fraction B. Since fractions A and B were extensively dialyzed before the assays, such a putative activator or co-factor would have to be a macromolecule of at least 10 kDa molecular mass.

With the aim to test this latter hypothesis, the T_7-tRNA substrate (ATP labelled) was first incubated for 6 h with SAM and enough tRNA(A-57) methyltransferase present in fraction B to

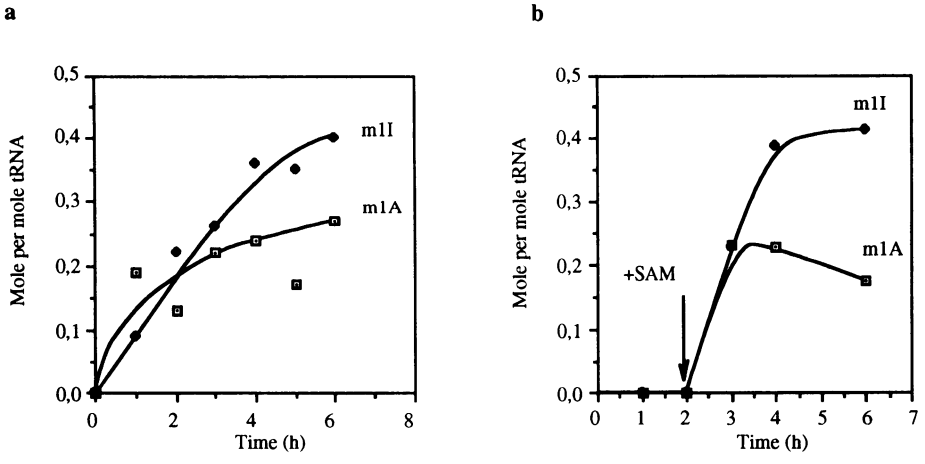

Figure 4. Time course of 1-methyladenosine (m^1A) and 1-methylinosine (m^1I) formation in *H.volcanii* synthetic $tRNA^{Ile}$ with the *H.volcanii* crude extract (S100) at 37°C in the presence of 2.2 M KCl. SAM (SAM) was supplemented at time 0 (**a**) or after 2 h incubation (**b**).

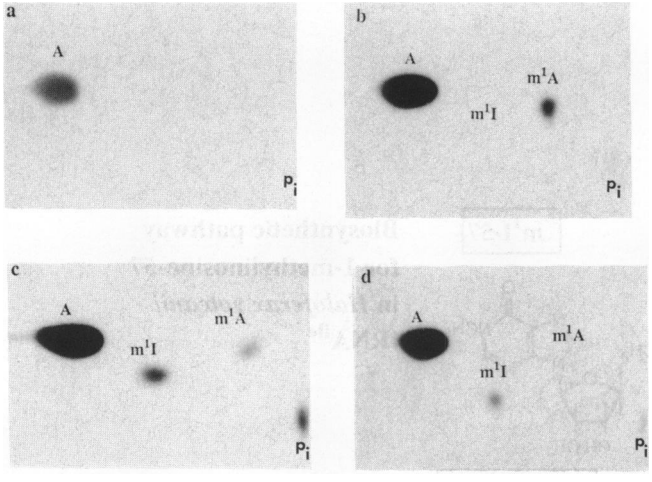

Figure 5. Autoradiograms of modified nucleosides formed in $tRNA^{Ile}$ transcript (wild type). The incubations were for 6 h at 37°C with fraction A and/or B obtained after chromatography of *H.volcanii* S100 extract on Sepharose 4B and supplemented with SAM. tRNA was labelled with [α-^{32}P]-ATP, hydrolyzed with nuclease P_1 and chromatographed using system I. (**a**) Incubation was with fraction A alone. (**b**) Incubation with fraction B alone. (**c**) Incubation was with a mix of fractions A and fraction B. (**d**) *H.volcanii* synthetic $tRNA^{Ile}$ was first modified with fraction B, then re-isolated by phenol-ethanol purification and further incubated for 6 h with fraction A.

obtain a modified $tRNA^{Ile}$ that contains almost 0.8 mol of m^1A-57 per mol of tRNA (Figs 5b and 6a). During incubation, <0.02 mol of m^1I-57 per mol of tRNA was formed. At the end of the incubation period, the reaction mixture was phenol-chloroform extracted twice and the nucleic acid precipitated with ethanol. The deproteinized m^1A-57-containing $tRNA^{Ile}$ was subsequently incubated for an additional 6 h in the absence of SAM and with fraction A only. As clearly shown in Figures 5d and 6b, m^1I-57 was quantitatively formed at the expense of m^1A-57. Thus, the enzyme catalyzing the formation of m^1I-57 from m^1A-57 in $tRNA^{Ile}$ of *H.volcanii* was present in fraction A. Because this reaction occurs without addition of any cofactor except salts, we conclude that this enzyme is probably a new kind of tRNA deaminase.

DISCUSSION

In this paper we have used an *in vitro* system in order to detect the activities of several tRNA modification enzymes of the archaebacterium *H.volcanii*. This assay, after optimization of the experimental conditions, will be used for further studies concerning the characterization of the various individual tRNA modification enzymes as well as the search of their identity elements in tRNAs.

Here we have focused on the enzymatic formation of the characteristic 1-methylinosine-57 that is present in almost two-thirds of the tRNAs sequenced so far from various archaea (mostly halophiles), the other tRNAs having in place a guanosine-57. The biosynthesis of this modified nucleoside is a two-step process that is catalyzed by separable enzymes (Fig. 7, upper part). The first step corresponds to a SAM-dependent methylation of A-57 into m^1A-57, catalyzed by a tRNA (A-57) methyltransferase. This enzyme may be analogous to those present in eubacteria and eukaryotes catalyzing the formation of m^1A at position 58 (not 57) in several tRNAs. Such enzymes have been already purified from *Thermus thermophilus* (36) and rat liver (37). Interestingly, m^1I-57 was found to be present adjacent to m^1A-58 in the archaeal initiator $tRNA^{Met}$ from *Thermoplasma acidophilum* and from *Sulfolobus acidocaldarius* (24). If the m^1I-57 in tRNA of these thermophilic organisms is formed by the same biosynthetic pathway as for m^1I-57 in *H.volcanii* tRNAs, then it would be interesting to determine whether the intermediate product m^1A-57 is formed by a methylase that is distinct from the one catalyzing the formation of m^1A-58 in the same tRNA molecule.

In the absence of SAM, inosine-57 could not be detected in our experimental conditions, even after very long incubation times. This observation suggested that m^1A-57 is an obligate intermediate of the two-step enzymatic reaction leading to m^1I-57 in *H.volcanii* tRNAs. The proof came from the fact that an enzyme able to use m^1A-57-containing tRNA, distinct from the one that catalyzes the formation of this intermediate, was present in fraction A eluted from a Sepharose 4B column. This enzyme apparently requires no co-factors and thus probably catalyzes the

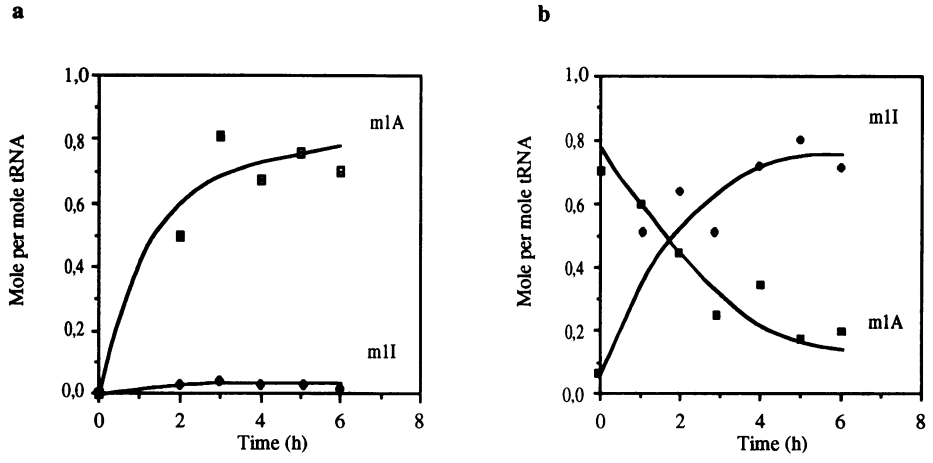

Figure 6. Time course of 1-methyladenosine (m^1A) and 1-methylinosine (m^1I) formation in *H.volcanii* $tRNA^{Ile}$. (**a**) [^{32}P]-ATP-labelled $tRNA^{Ile}$ was incubated with fraction B at 37°C in the presence of SAM. After 6 h, the incubation mixture was phenol-extracted, ethanol-precipitated and the recovered $tRNA^{Ile}$ was added to a new incubation mixture containing fraction A in the absence of SAM (**b**). Analysis of the modified nucleotides was as described in Materials and Methods.

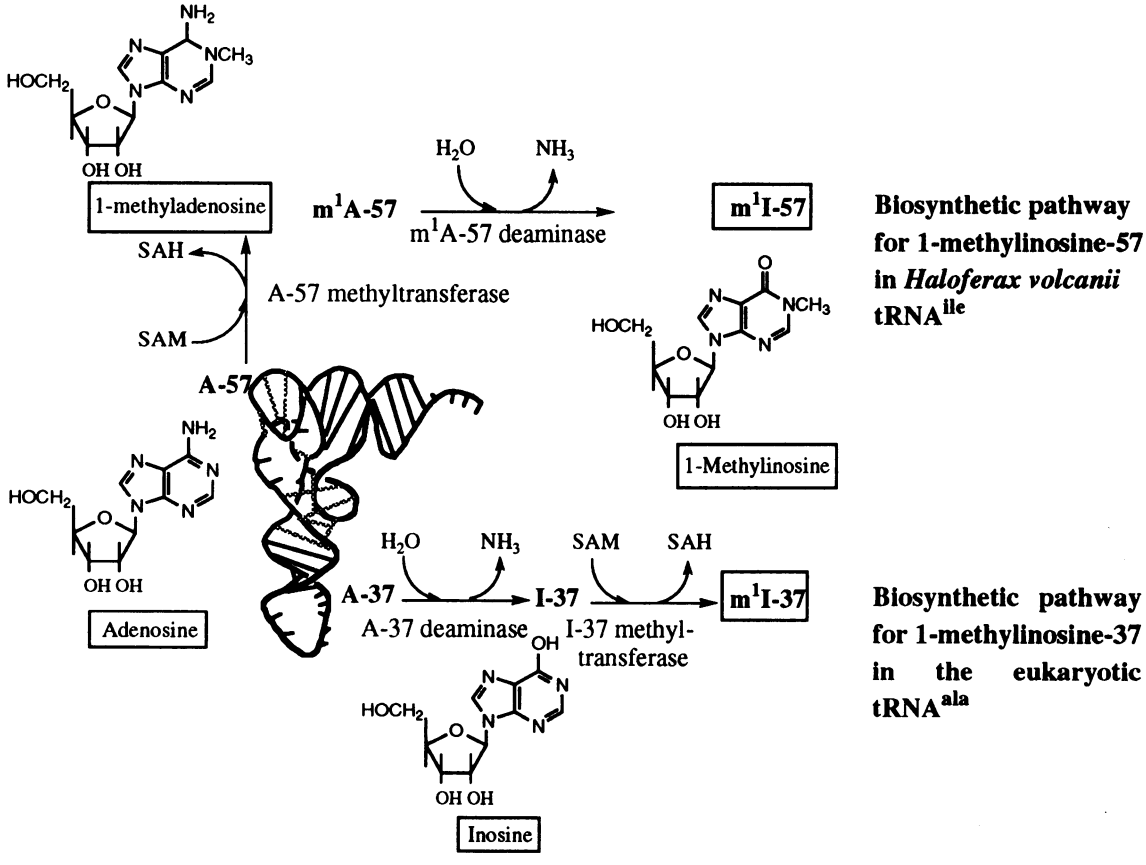

Figure 7. Comparison of the biosynthetic pathways for 1-methylinosine-57 in *H.volcanii* $tRNA^{ile}$ and 1-methylinosine-37 in the eukaryotic $tRNA^{Ala}$. A: adenosine; m^1A, 1-methyladenosine; m^1I, 1-methylinosine; SAM: S-adenosylmethionine; SAH, S-adenosylhomocysteine.

hydrolytic deamination of 1-methyladenosine-57 by a mechanism which could be similar to those assigned to ADA (38, reviewed in 39), to double-stranded RNA adenosine deaminase (dsRAD) (40) and to tRNA(A-34) deaminase (39, reviewed in 40). One essential difference, however, is that m^1A instead of a non-methylated adenosine is the target of the *H.volcanii* deaminase. This methyl group on a purine nitrogen may strongly influence the mechanism of the deaminase reaction itself, since

the pKa (8.8) of 1-methyladenosine (43) is higher than the pKa (3.5) of adenosine, at least in the free state. Therefore, at variance with other deaminases acting primarily on free adenosine or on adenosine within an RNA, one could anticipate that there might be no need of zinc in the active site of the *H.volcanii* tRNA(m^1A-57) deaminase. Indeed, in the case of the adenosine desaminase (ADA) acting on free adenosine, a zinc atom has been demonstrated to play a major role in the activation of a bound water molecule, presumably to a hydroxide ion, which attacks the carbon-6 adjacent to N-1 to generate a tetrahedral intermediate, before the release of the NH_3 group (38,39).

Alternatively, the possibility also exists that the methyl group on N-1 of adenosine-57 is required as an essential identity element for the recognition of the target base by the *H.volcanii* tRNA (m^1A-57) deaminase. Noteworthy, a strict requirement of a modification on one atom for a second modification to occur on an other atom of the same target base have been demonstrated to date only in the case of sequential enzymatic reactions leading to ms^2i^6A-37 formation in tRNA. In this latter case, enzymatic isopentenylation of C-6 is strictly required for subsequent enzymatic thiolation and methylation of C-2 of adenosine-37 (reviewed in ref. 44).

Whatever the molecular details of the two-step process of m^1I-57 formation in tRNAs of *H.volcanii*, the biochemical pathway is definitely different from that leading to the enzymatic formation of m^1I at position 37 of the anticodon of eukaryotic $tRNA^{Ala}$ (see Fig. 7, lower part) (28, Simon and Grosjean, unpublished results).

Lastly, enzymatic digests of tRNAs from the extremely thermophilic archaebacteria *Pyrodictium occultum* (optimal growth 105°C), *Pyrodictium islandicum* (100°C) and *Thermococcus MSB4* (88°C) contain 1,2′-O-dimethylinosine (m^1Im) (6,11). Its location in tRNAs has not yet been established on the basis of sequence analysis. However, the probability is high that it corresponds to the 2′-O-methylated form of the same m^1I-57 that we have studied above. If confirmed, this raises the following questions: (i) is the pathway for enzymatic formation of m^1I-57 in hyperthermophilic archaea the same as for m^1I-57 in extremely halophilic archaea?; (ii) does the methylation of the 2′OH of the sugar occur before or after the methylation of the base?; (iii) how do these base and ribose modifications influence the stability, and biological functions of this important class of RNA molecules?

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